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Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment

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A B S T R A C T

Pretreatment technologies are aimed to increase enzyme accessibility to biomass and yields of fermentable sugars. In general, pretreatment methods fall into four different categories including physical, chemical, physico-chemical, and biological. This paper comprehensively reviews the lignocellulosic wastes to bioethanol process with a focus on pretreatment methods, their mechanisms, advantages and disadvantages as well as the combinations of different pretreatment technologies. Moreover, the new advances in plant “omics” and genetic engineering approaches to increase cellulose composition, reduce cellulose crystallinity, produce hydrolases and protein modules disrupting plant cell wall substrates, and modify lignin structure in plants have also been expansively presented.

Contents

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1. Introduction

Recent economic developments in many countries all around the globe have heightened the need for alternative energy resources due to the well-documented drawbacks of fossil fuels: (1) their finite supply (2) greenhouse gasses emission and global warming and (3) increasing price and unexpected fluctuations. All these weaknesses have strengthened the interest in alternatives, renewable, sustainable, and economically viable fuel such as bioethanol. Bioethanol can be either mixed with gasoline or used as a sole fuel using dedicated engines; moreover, it has higher heat of vaporization and octane number compared to gasoline [1]. Ethanol is already blended with gasoline and supports by vehicle manufacturers have resulted in vehicles that can use up to an 85% ethanol–15% gasoline mixture [2]. In fact, gasoline can use bioethanol as an oxygenated fuel to increase its oxygen content, causing better hydrocarbon oxidation and diminishing greenhouse gasses [3].

In the first generation bioethanol production, expensive starch and sugar derived from sugar cane and maize are employed as feedstock but in the second generation process, lignocellulosic materials, which are cheap, abundant and renewable, are used [4]. Besides, lignocellulosic materials do not negatively affect the human food supply chain by eliminating the food in favor of bioethanol production [5]. Lignocelluloses are composed of cellulose, hemicelluloses and lignin (Fig. 1) in an intricate structure, which is recalcitrant to decomposition. One of the best strategies to convert such biomass into sugars is enzymatic saccharification due to its low energy requirement and less pollution caused; but, the major problem is the low accessibility of cellulose because of rigid association of cellulose with lignin [6]. This leads to difficulties within the conversion process; therefore, breaking down lignin seal in order to make cellulose more accessible to enzymatic hydrolysis for conversion is one main aim of pretreatment (Fig. 2). In other words, pretreatment is the crucial and costly unit process in converting lignocellulosic materials into fuels [7].

A suitable pretreatment procedure involves (1) disrupting hydrogen bonds in crystalline cellulose, (2) breaking down coross-linked matrix of hemicelluloses and lignin, and finally, (3) raising the porosity and surface area of cellulose for subsequent enzymatic hydrolysis [8,9]. There are several pretreatment methods including, physical pretreatment (grinding and milling, microwave and extrusion), chemical pretreatment (alkali, acid, organosolv, ozonolysis and ionic liquid), physico-chemical pretreatment (steam explosion, liquid hot water, ammonia fiber explosion, wet oxidation and CO_2 explosion) and biological pretreatment. On the other hand, regrdless of the pretreatment method used, some inhibitory compounds are produced during the process, which have negative effects on microbial activity in the hydrolysis step. Inhibitors are classified into three major groups: (1) weak acids such as levulinic, acetic and formic acids, (2) furan derivatives such as HMF (5-hydroxy-2-methyll furfural) and furfural as well as (3) phenolic compounds [10].

The purpose of this paper is to review different pretreatment methods for bioethanol production and to offer an in-depth discussion on the benefits and drawbacks of each while striving to present and highlight several combined pretreatment methods. Moreover, the crucial role of genetic and metabolic engineering in facilitating pretreatment and hydrolysis processes and consequently in economical production of ethanol from lignocellulosic wastes has been also discussed.

2. Lignocellulosic material composition

2.1. Cellulose

Cellulose (C6H10O5)*x*, the main constituent of lignocellulosic biomass, is a polysaccharide that consists of a linear chain of D-glucose linked by β-(1,4)-glycosidic bonds to each other. The Cellulose strains are associated together to make cellulose fibrils.

Cellulose fibers are linked by a number of intra- and intermolecular hydrogen bonds [12]. Therefore, cellulose is insoluble in water and most organic solvents [13].

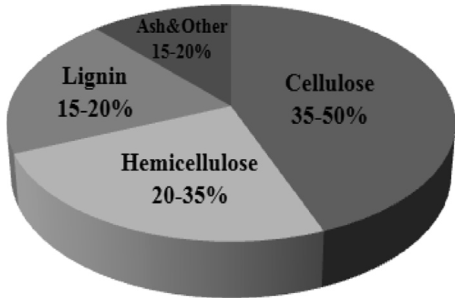


Fig. 1. General composition of lingocellulosic biomass feedstock.

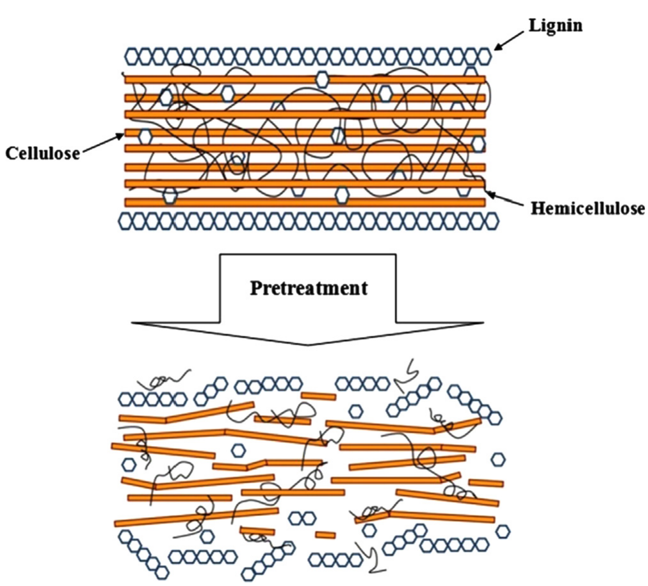


Fig. 2. Schematic pretreatment of lignocellulosic material.

2.2. Hemicelluloses

Hemicelluloses (C5H8O4)*m*, located in secondary cell walls, are heterogeneous branched biopolymers containing pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D galactose) and/or urgonic acids (α-D-glucuronic, α-D-4-O-methyl-galacturonic and a-D-galacturonic acids) [14]. They are relatively easy to hydrolyze because of their amorphous, and branched structure (with short lateral chain) as well as their lower molecular weight [12]. In order to increase the digestibility of cellulose, large amounts of hemicelluloses must be removed as they cover cellulose fibrils limiting their availability for the enzymatic hydrolysis [15]. Hemicelluloses are relatively sensitive to operation condition, therefore, parameters such as temperature and retention time must be controlled to avoid the formation of unwanted products such as furfurals and hydroxymthyl furfurals which later inhibit the fermentation process [10].

2.3. Lignin

Lignin [C9H10O3(OCH3)0.9–1.7]*n* is an aromatic polymer synthesized from phenylpropanoid precursors. The major chemical phenylpropane units of lignin consisting primarily of syringyl, guaiacyl and p-hydroxy phenol are linked together by a set of linkages to make a complicated matrix [16].

3. Influence of lignocellulosic biomass composition and structure on cellulose hydrolysis and bioconversion

As mentioned earlier, lignocellulosic biomass composition plays a very crucial role in the performance and efficiency of both pretreatment and biodegradation stages. Table 1 presents the compositions of several suitable lignocellulosic biomass for bioethanol production [8,17–27]. Utilization of cellulose in native form, not only consumes large amount of enzyme but also results in low cellulose enzymatic digestibility yield ($<20\%$). Therefore, some structural modification of lignocellulosic material (pretreatment), is required and to select a suitable pretreatment technology, recognition of the main structural limiting factors is a critical step. These factors include (1) specific surface area, (2) cellulose crystallinity index (CrI), (3) degree of polymerization (Dp),

Table 1
Different lignocellulosic biomass compositions (% dry basis).

Lignocellulosic biomass	Cellulose glucan	**Hemicellulose**				**lignin**		**References**
		Xylan	Arabinan	Galactan	Mannan	Acid-insoluble lignin	Acid-soluble lignin	
Barley hull	33.6	30.5	6.1	0.6	Trace	19.3	ND	[17]
Barley straw	33.8	21.9				13.8		[18]
Corn cobs	33.7	31.9				6.1		[18]
Corn stover	38.3	21.0	2.7	2.1	ND	17.4		[19]
Cotton stalks	14.4		14.4			21.5		[18]
Wheat straw	30.2	18.7	2.8	0.8	ND	17		[20]
Rice straw	31.1	18.7	3.6	ND	ND	13.3		[21]
Rye straw	30.9	21.5	ND	ND	ND	22.1	3.2	[22]
Oat straw	39.4	27.1				17.5		[18]
Soya stalks	34.5	24.8				9.8		[18]
Sunflower stalks	42.1	29.7				13.4		[18]
Switchgrass	39.5	20.3	2.1	2.6	ND	17.8	4.0	[8]
Sugarcane bagasse	43.1	31.1				11.4		[23]
Sweet sorghum bagasse	27.3	13.1	1.4	ND	ND	14.3		[24]
Forage sorghum	35.6	18.4	1.8	ND	ND	18.2		[24]
Olive tree pruning	25.0	11.1	2.4	1.5	0.8	16.2	2.2	[25]
Poplar	43.8	14.8	ND	ND	ND	29.1		[26]
Spruce	43.8	6.3	ND	ND	14.5	28.3	0.53	[27]
Oak	45.2	20.3	ND	ND	4.2	21.0	3.3	[27]

(4) cellulose sheathing by hemicelluloses, (5) lignin content and (6) acetyl content [28].

4. Pretreatment methods

4.1. Physical pretreatment

The objective of physical pretreatment such as milling, grinding, chipping, freezing, radiation is to increase surface area and reduce particle size of lignocellulosic materials [29]. Moreover, it leads to decrease degree of polymerization and decrystallization of feedstock. Combination of physical pretreatments and other pretreatment is usually used.

4.1.1. Milling

Milling is usually considered as the first step of pretreatment. Ball milling, two-roll milling, hammer milling, colloid milling and disk milling are several types of milling used in bioethanol production processes. The final particle size achieved depends on the type of the physical pretreatment, for example, after chipping, milling or grinding, the particle size reduces to 10–30 mm and 0.2–2 mm, respectively [30]. The most important drawback of milling is high energy requirement. To overcome that, wet disk milling has been introduced as a more reasonable mechanical pretreatment in terms of energy consumption. However, the yields of glucose and xylose after enzymatic hydrolysis of some pretreated biomass by ball milling are higher than those of wet disk milling. For instance, Hideno et al. investigated the enzymatic hydrolysis of rice straw after being pretreated by wet disk milling and ball milling. They reported the yields of glucose and xylose of 78.5% and 41.5%, and 89.4% and 54.3%, respectively.

Sant'Ana da Silva et al. evaluated the effectiveness of ball milling and wet disk milling on treating sugarcane bagasse. Based on their investigation, glucose and xylose hydrolysis yields under optimum conditions for ball milling-treated bagasse were 78.7% and 72.1% respectively, while maximum glucose and xylose yields for bagasse wet milling-treated were 49.3% and 36.7%, respectively [31,32].

4.1.2. Extrusion

Extrusion pretreatment is a thermo-physical pretreatment in which materials undergo mixing, heating and shearing leading to physical and chemical alteration [5,33]. High shear, rapid mixing, short residence time, moderate barrel temperature, no furfural and HMF formation, no washing and conditioning, adaptability to process modification, easy scale-up, and most importantly, possibility of continuous operation are considered among the advantages of this method [34,36]. Furthermore, extrusion results in no effluent and consequent effluent disposal cost and solid loss [35]. Yoo et al. [33] employed extrusion pretreatment (screw speed, 350 rpm; maximum barrel temperature, 80 °C; in-barrel moisture, 40% wet basis) for soybean hulls and achieved 94.8% glucose conversion after enzymatic hydrolysis (glucose yield of 0.37 g/g biomass).

4.1.3. Microwave

Microwave irradiation could be an alternative to the conventional heating in order to alter the ultra structure of cellulose, degrade/partially remove lignin and hemicelluloses, disrupt the silicified waxy surface and finally enhance the enzymatic susceptibility of reducing sugars [37,38]. Technically, conventional heating is based on superficial heat transfer but, as for microwave, heat is generated by direct interaction of a heated object and an applied electromagnetic field [39]. More specifically, microwave irradiation leads to cellulosic breakdown mainly through molecular collision due to dielectric polarization [40]. The advantages of this method include, (1) short process time, (2) high uniformity and selectivity (3) and less energy input than the conventional heating [41].

4.1.4. Freeze pretreatment

A novel approach recently developed for physical pretreatment of biomass is the freeze pretreatment and has been found to significantly increase the enzyme digestibility of rice straw. In spite of the high cost involved and that only a few studies have been carried out using this pretreatment so far, its unique features i.e. lower negative environmental impact, application of less dangerous chemicals and high effectiveness have attracted a great deal of attention [42].

4.2. Chemical pretreatment

4.2.1. Acid pretreatment

Acid pretreatment in particular by using sulfuric acid is the most commonly employed chemical pretreatment for lignocellulosic biomass where polysaccharides (mainly hemicelluloses) are hydrolyzed to monosaccharides leading to higher accessibility of cellulose to enzyme hydrolysis. Acid pretreatment can be performed either under low acid concentration and high temperature or under higher acid concentration and lower temperature. [43]. Using concentrated acid is more economic as the process is performed at low temperature [14]; however, toxicity, corrosiveness of equipments, acid recovery [30], degradation of monosaccharides i.e. glucose, and the production of fermentation inhibitors (such as furan-type inhibitors; HMF (5-hydroxy methyl- furfural) and 2-furfuralaldehyde [44,45]), are major drawbacks preventing the widespread application of this method [5,]. Moreover, at high temperatures, the produced inhibitors such as furfural could also degrade into other unwanted products e.g. formic and levulinic acids [46]. Industrially, diluted acid is more attractive as it generates lower amounts of fermentation inhibitors and in light of that, numerous studies have been conducted employing this technique. For instance, rice straw was pretreated with 1% (w/w) sulfuric acid with 1–5 min retention time at 160° or 180 °C followed by enzymatic hydrolysis, which resulted in the maximal sugar yield of 83% [47]. In a different investigation using the same acid, rape seed straw with 20% solid content was pretreated for 10 min at 180 °C and 75.12% of xylan and 63.17% of glucan were respectively converted into xylose and glucose through enzymatic hydrolysis [48]. Redding et al. [2] also pretreated Coastal Bermuda grass with sulfuric acid (1.2%) at 140 °C for 30 min and total sugar yield of 94% of the theoretical value was achieved.

Another acid used is peracetic acid which is capable of converting lignin to soluble fragments [49]. However, it is explosive in concentrated form and costly as well [50]. Rocha et al. [51] applied a mixture of sulfuric and acetic acid to pre-treat sugarcane bagasse. The solution used contained 1% (w/v) sulfuric acid and 1% (w/v) acetic acid. Solid-to-liquid ratios tested were 1.5:10 and 1:10 and the pretreatment was performed in a rotary reactor for 10 min at 190 °C. The outcome for conditions was efficient removal of hemicellulose by above 90% and cellulose degradation was recorded below 15%. In addition to inorganic acids, organic acids such as maleic and fumaric acids could also be used. In a study, hydrolysis yields when wheat straw was treated by maleic and fumaric were compared with that of sulfuric acid and it was revealed that wheat straw could be pretreated efficiently with organic acids as well [52].

4.2.2. Alkaline pretreatment

Removing lignin, acetyl groups and different uronic acid substitution which inhibit the cellulose accessibility for enzymatic

saccharification are the most crucial advantages of this pretreatment [19]. Solubilization of hemicelluloses and cellulose in this method is less than in acid or hydrothermal processes [53]. This method is also known for causing chemical swelling of fibrous cellulose [7], in which, saponification and salvation reactions occur which lead to the disruption the crosslinks between hemicelluloses and the other components, hence, increasing the porosity of biomass [30]. More specifically, the ester bond cross-linking lignin and xylan are disrupted leading to delignification [54]. Comparatively, alkaline pretreatment is operated at lower temperatures [55] and does not require complex reactors, which are appealing to be employed on-farm [56]. However, the major drawbacks are long residence time (from hours to days) and the need for neutralization of the pretreated slurry [12,57]. Sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($CaOH_2$), and ammonia are mostly used in this pretreatment method. Wan et al. [57] pretreated soybean straw with NaOH (4–40 g/100 g dry straw) at ambient condition and achieved glucose yield of 64.55% and xylan removal of up to 46.37%. In another study, Costal Bermuda grass was pretreated with 0.75% NaOH solution during 15 min, and a total reducing sugar yield of 71% was achieved. In addition, the overall conversion efficiencies for glucan and xylan were 90.43% and 65.11%, respectively [59].

Combination of NaOH and$CaOH_2$ (0.10 and 0.02 g/g raw biomass, respectively), were also used in a study to enhance the cost effectiveness of alkaline pretreatment of switchgrass at ambient condition. Biomass was first pretreated by NaOH, and the regenerated biomass was then pretreated by $Ca(OH)_2$ which led to glucose and xylose yields of 59.4% and 57.3%, respectively [58].

4.2.3. Ionic liquid (IL) pretreatment

Ionic liquids (ILs)-based pretreatment of lignocellulosic biomass has gained much attention recently and they can dissolve carbohydrates and lignin simultaneously. ILs (melting point < 100 °C [60]), are organic salts composed of cations and anions, typically, large organic cations and small inorganic anions [5]. In fact, cation structure (the symmetry and the length of alkyl substituents, the presence of hydrophobic groups, etc.) and degree of anion charge delocalization are two major factors that can affect physical, chemical and biological properties of ILs. Overall, cations, anions, temperature, and time used in the pretreatment process are the major factors affecting the interaction between the ILs used and the lignocellulosic materials [61]. Cellulose could be dissolved by ILs containing chloride, formate, acetate or alkylphosphonate inions by formation of strong hydrogen bonds [65]. In another word, hydrogen bonds are formed between the non-hydrated ions of ILs and the sugars' hydroxyl protons leading to the degradation of the complex network of cellulose, hemicelluloses and lignin [5]. Among ILs investigated, 1-allyl-3-methylimidazolium-chloride ([AMIM]Cl), 1-ethyl-3-methylimidazolium-acetate ([EMIM]Ac), 1-butyl-3-methylimidazolium chloride ([BMIM]Cl) and 1-ethyl-3-methyl imidazolium diethyl phosphate ([EMIM]DEP) have recently received much attention due to their remarkable cellulose dissulotion capability [66].

In a study, pretreatment of switchgrass using [EMIM]Ac produced a glucan content ranging from 50.4% to 67.7%. This IL was also found capable of removing 69.2% of total switchgrass lignin at 160 °C for 3 h [8]. [BMIM]Cl has also been reported as one of the best cellulose solvent (up to 25 wt% of cellulose) [13]. In a different study on oil palm frond (OPF) pretreatment using [BMIM]Cl, cellulose crystallinity reduction and improved enzymatic digestibility was accomplished [60]. As for lignin, 1-butyl-3-methylimidazolium trifluoromethanesulfonate and 1,3-dimethylimidazolium methylsulphate have been reported as the best solvents [67].

Although significant changes in biomass structure after ILs pretreatment have been reported but composition of biomass was proven to alter slightly [60]. For example, lignin, hemicellulose and cellulose percentages in OPF before and after IL treatment were measured at 27, 36, 37 wt%, and 27, 34, 39 wt%, respectively [60]. This could be explained by the fact that after ILs pretreatment, regenerated cellulose becomes amorphous and porous, which is much more susceptible to degradation by cellulases [62]. The ILs' advantages in comparison with the other conventional methods include, less dangerous process condition and chemicals, remaining liquid in a wide range of temperature, being green solvents due to low vapour pressure and finally high thermal and chemical stability [60]. Besides, ILs are non-derivatizing [13], require mild operational conditions; and, ionic liquids are easily recycled. On the other hand, incompatibility of IL with cellulase leading to the inactivation and unfolding of the enzyme is regarded as one of their most important disadvantages [64]. Viscosity is of great importance when applying ILs as low viscosity causes cellulose to be dissolved at low temperature thus lowering energy consumption. Moreover, high temperatures cause some negative side-effects, such as deteriorated stability of ILs, and occurrence of side-reactions [63].

Application of ILs at industrial scale is faced with three major challenges: (1) huge amounts of currently expensive ILs is needed, (2) recycling of pure ILs is energy-intensive and, (3) during pretreatment, solution becomes viscous that makes it difficult to handle [68]. Fu and Mazza [68] offered a strategy to overcome these problems which was the application of water-mixtures of ILs. They managed to reduce the amount of ILs required which in turn decreased viscosity and, finally IL was recycled easily as evaporation or reverse osmosis were not necessary.

4.2.4. Organosolv pretreatment

Organic solvents i.e. methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl alcohol, with or without the addition of a catalyst agent could be used in organosolv process [70]. The catalysts used include organic or inorganic acids (hydrochloric and sulfuric acids), bases (sodium hydroxide, ammonia and lime) [69]. This pretreatment is capable of breaking the internal lignin and hemicelluloses bonds and is therefore especially efficient for high lignin lignocellulosic biomass. Moreover, relatively pure and high-quality lignin can be obtained as a by-product through this process [70]. Obviously, lignin removal leads to an increased surface area making cellulose more acceceble to enzyme [71]. The main drawbacks of this method are low-boiling point of organic solvents, high risk of high-pressure operation, and flammability and volatility of such solvents [72]. Moreover, solvents should be recycled in order to diminish the operation cost and prevent their inhibitory effects on enzymatic hydrolysis and microorganisms [30]. Koo et al. [71] treated *Liriodendron tulipifera* with ethanol, and sodium hydroxide as catalyst and managed to minize the loss of glucan. In a different investigation, *Pinus radiata* was exposed to acetone–water pretreatment (acetone: water molar ratio of 1:1) at 195 °C, pH 2.0 for 5 min and ethanol yield of about 99.5% was achieved [73].

4.2.5. Ozonolysis

This pretreatment includes using ozone gas as an oxidant in order to break down lignin and hemicelluloses and increase cellulose biodegradability [74]. Being a powerful oxidant, soluble in water and available, are major advantages of ozone gas. It also could break down lignin and release soluble compounds of less molecular weight such as acetic and formic acid. Williams [75] achieved lignin degradation of 49% when corn stover was broken down by ozonolysis. His findings were later confirmed by those of

Garcia-cubero et al. [22] where enzymatic hydrolysis yields of up to 88.6% and 57% were obtained compared to 29% and 16% in non-ozonated wheat and rye straw respectively. The most important advantage of this method is lack of degradation by-products which leads to less complication in the subsequent hydrolysis steps [22]. Besides, this process is carried out at ambient condition. On the other hand, the main disadvantage is the cost of ozone used as a large amount of ozone is employed to treat lignocellulosic materials [30].

4.3. Physico-chemical pretreatment

4.3.1. Steam explosion pretreatment

This is an extensively investigated thermo-mechanochemical method which involves the break-down of structural components by steam-heating (thermo), shearing (mechano; due to sudden decompression and evaporation of moisture), and auto-hydrolysis (chemical) of glycosidic bonds. More specifically, biomass particles are heated using pressurized steam (20–50 bar, 160-270 °C) for several seconds to a few minutes and then the pressure is released to atmospheric pressure, condensed moisture evaporates and desegregation of lignocellulosic matrix takes place [76,]. It is believed that this pretreatment causes hemicellulose hydrolysis, lignin transformation due to high temperature and increases crystallinity of cellulose by promoting crystallization of the amorphous portions.

The parameters affecting steam explosion efficiency are particle size, temperature (T) and residence time (t) and the combined effect of both temperature and residence time is described by severity factor (R_0) [77]:

$$(\log R_0)\ R_0 = \int_0^t \exp\left[\frac{T-100}{14.75}\right] \mathrm{d}t$$

The hydrolysis of hemicellulose during steam explosion pretreatment is accomplished by organic acids such as acetic acids generated from hydrolysis of acetyl groups associated with the hemicellulose and formic and levulinic acids derived from other functional groups. Water, also acts as an acid and possesses certain acid properties at high temperature. Finally, hemicellulose removal from the surface of cellulose microfibrils increases enzyme accessibility and enzymatic hydrolysis rate of cellulose by exposing the cellulose surface [78]. However, due to the existing acidic conditions, the degradation of sugars into furfural and HMF might also happen during the process [79].

For some lignocellulosic biomass e.g. softwood in in order to reach high sugar yields, addition of an acid catalyst such as H_2SO_4 or SO_2 is a prerequisite to the steam explosion process. This is ascribed to the low content of acetyl groups in the softwood's hemicellulose. On the other hand, this also leads to the formation of higher amounts of inhibitory compounds which would adversely affect the reaction rate during the fermentation process [80]. Therefore, lignocellulosic biomass needs to be washed by water after pretreatment in order to remove these inhibitors.

In a study, Reczey and Zacchi [81] reported that steam explosion pretreatment at 200 °C for 5 min after swelling the fibers (Corn Stover) with 2% sulfuric acid enhanced the enzymatic conversion (50 °C, 24 h) of cellulose to glucose by nearly four times at 80%, compared to the untreated raw materials and that the ethanol yield of about 90% was achieved. In a different experiment, the optimal conditions for steam explosion pretreatment (with 0.9% sulfuric acid as catalyst) of wheat straw which led to the maximum overall sugar yield of 85% of total sugars contained in raw material were found at 180 °C and 10 min [20]. Steam explosion due to its lower capital investment and higher energy efficiency is among the very limited number of cost-effective pretreatment technologies for pilot scale demonstration and commercialized applications [77].

4.3.2. Ammonia fiber explosion (AFEX) pretreatment

In AFEX, pretreatment of biomass is conducted by using liquid ammonia and based on the steam explosion process concept. Four parameters including ammonia loading, water loading, reaction temperature, and residence time can be varied in order to optimize the AFEX pretreatment [82]. The process includes high pressure (1.72–2.06 Mpa) and moderate temperatures (60–120 °C) for several minutes ($<$30 min) followed by a sudden pressure release [83]. Rapid expansion of the ammonia gas causes cleavage of lignin-carbohydrate complex and consequent physical disruption of biomass fibers leading to increased digestibility of biomass [84].

In contrast with steam explosion that produces a slurry, AFEX due to the ammonia's low boiling point, produces only solid material. It also does not liberate any sugars directly because of low hemicelluloses solubilizetion but opens up the structure of lignocellulosic biomass and increases polymers surface area and consequently, the enzymatic digestibility. AFEX pretreatment has been demonstrated to result in higher conversion rates of different kinds of cellulosic biomass, such as aspenwood, wheat straw, alfalfa stems [85] switchgrass [86], rice straw [87], corn stover [88], and has been shown to be less effective on high-lignin content biomass such as hardwood and softwood feedstocks.

Numorous studies have been conducted to determine the optimal AFEX conditions for different biomas sources. Li et al. [24] reported ammonia to biomass loading of 2:1, 120% moisture (dry weight basis), 140 °C and residence time of 5 min as optimal to convert both forage and sweet sorghum bagasse to ethanol. As for poplar (*Populus nigra*) and corn stover Balan et al. [89] found optimal conditions as 2:1 ammonia to biomass loading, 233% moisture (dry weight basis), and 180 °C and 1:1, ammonia to biomass loading, 60% moisture, and 90 °C, respectively. Overall, in order to reduce the high operation cost basically due to the high cost of ammonia as well as environmental issues, an efficient ammonia recovery and recycling seems to be inevitable [90].

4.3.3. CO_2 explosion pretreatment

Basically, super-critical CO_2 explosion is the same as AFEX and steam explosion pretreatments as CO_2 molecules have a similar size property to those of water and ammonia making them capable of penetrating into small pores of lignocellulosic material [92]. In contrast with steam explosion, supercritical CO_2 explosion needs lower temperature and is also less costly in comparison with AFEX [92], making it an ideal choice among the explosion-type methods. Besides, CO_2 explosion possesses other advantages such as non-toxicity and non-flammability.

Super critical CO_2 (critical temperature (Tc) of 31 °C and critical pressure (Pc) of 7.4 MPa) possesses a liquid-like density while it exhibits gas-like transport properties of diffusivity and viscosity [91]. On the other hand, while CO_2 pretreatment maintains the advantages of an acid-catalyzed process by forming carbonic acid when CO_2 is dissolved in water, but due to the specific features of carbonic acid, it leads to significantly less corrosiveness. Furthermore, due to the easy removal of CO_2 by depressurization, it creates no waste products and requires no further recovery [93].

Kim and Hong [94] evaluated the effect of supercritical CO_2 pretreatment on enzymatic digestibility of aspen wood and southern yellow pine with a 73% (w/w) moisture content. They argued that aspen and southern yellow pine pretreated with supercritical CO_2 at 21.37 Mpa and 165 °C for 30 min in comparison with the untreated ones produced higher amounts of reducing sugar (form 14.5 and 12.8% to 84.7 and 27.3%, respectively) when underwent the enzymatic hydrolysis process.

4.3.4. Liquid hot water (LHW)

LHW pretreatment employs high temperatures (160–220 °C) and pressure to keep water in liquid state and in contact with biomass for about 15 min residence time without addition of any chemicals or catalysts. In LHW and unlike steam explosion, rapid decompression or expansion is not required and utilization of pressure is only for maintaining water and preventing evaporation. LHW pretreatment has been shown as an efficient method for treating different kinds of lignocellulosic material, including corncobs [95], sugarcane bagasse [96], corn stover [97], wheat straw [98] and rye straw [99]. This method is reported to be capable of solubilizing most of the biomass hemicellulose by $>80\%$ [96], and consequently increasing cellulose digestibility through hemicelluloses removal [100]. The slurry generated through the pretreatment process consists of solid (enriched cellulose and water-insoluble materials) and liquid fraction (water and most of the solubilized hemicelluloses) and low or no inhibitors.

In order to prevent the formation of inhibitors and sugar degradation during the LHW, pH should be controlled between 4 and 7. Laser et al. [96] optimized the pH-controlled LHW pretreatment of corn stover (190 °C for 15 min) and achieved maximized hemicellulose solubilization while minimizing the formation of inhibitors. In addition, they managed to obtain cellulose to glucose conversion rate of 90% through the enzymatic hydrolysis. In a different study, Perez et al. [98] also employed LHW for treating wheat straw and reported similar findings under optimized condition when they accomplished considerable xylose and glucose yields of 80% and 91%, respectively.

4.3.5. Wet oxidation (WO)

Oxygen or air as catalyst and water are employed in WO pretreatment technology. This operation usually occurs at temperature above 120 °C and pressures ranging between 0.5 and 2 MPa for about <30 min [101]. It has been proven that WO is an efficient pretreatment technique for fractionation of lignocellulosic materials by solubilization and hydrolysis of hemicelluloses and delignification with organic acid formed during the pretreatment process and oxidative reactions. Formation of inhibitors such as furfural and HMF in comparison with steam explosion and LHW is lower in the WO pretreatment. Moreover, by using an alkali in combination with WO, increased monosaccharide sugars production and less inhibitory compounds generation due to decreased acidity have been reported [102].

The best WO pretreatment conditions for rice husk were achieved in a study conducted by Banerjee et al. [103] where they applied a reaction temperature of 185 °C and air pressure of 0.5 MPa for 15 min. They managed to maintain 66.97% of the cellulose in the solid fraction, while achieved 89% lignin removal. On the other hand, 69.77% of the hemicellulose was solubilized through the WO treatment. Similarly, the findings obtained by Martin et al. [23] found WO an appropriate method for fractionating and consequent enzymatic hydrolysis of sugarcane bagasse. In their investigation, alkaline WO (195 °C, 15 min) resulted in the highest yield of solid material with nearly 70% cellulose content, solubilization of approximately 93% of hemicelluloses and 50% lignin removal. Furthermore, an enzymatic convertibility of cellulose of around 75% was accomplished. Despite of WO's advantages, its economic application is generally ruled out due to the high capital cost imposed by pressure equipment and the high cost of oxygen and catalyst used.

4.4. Biological pretreatment

Unlike the chemical and physicochemical pretreatment methods, biological or microbial pretreatment has no chemical requirements. It is basically an environmental friendly pretreatment converting lignocellulosic biomass by microorganisms especially fungi into more accessible compounds for hydrolysis and subsequent bioethanol production [104]. In contrast to most of the pretreatment methods that require high capital and operational cost, this method only takes advantage of white-, brown-, soft-rot fungi to delignify and enhance enzymatic hydrolysis of lignocellulosic biomasses [105]. The highest efficiency among the biological pretreatment methods has been achieved by lignin-degrading white-rot fungi for the soft and brown fungi only attack cellulose. Among the known species of white-rot fungi used to date, the highest efficiency belongs to *Phanerochaete chrysosporium* due to its high growth rate and lignin biodegradation capabilities [106]. Shi et al. [103] studied the impact of white-rot fungi *P. chrysosporium*, on cotton stalks under two different culture conditions and reported 19.38% and 35.53% lignin degradation in submerged cultivation (SmC) and solid state cultivation (SSC).

In biological pretreatment, particle size, moisture content, pretreatment time and temperature could affect lignin degradation and enzymatic hydrolysis yield. Wan and Li [107] used *Ceriporiopsis subvermispora* for ethanol production on corn stover while investigating the effect of various factors on the pretreatment efficiency. The findings obtained revealed up to 31.59% lignin degradation while maintaining 94% of cellulose during an 18-d pretreatment. In addition, the highest glucose yield of 66.61%, was achieved at 28 °C with the moisture content 75% and the particle size of 5 mm. Various microbial agents could also significantly affect the pretreatment efficiency of certain biomass. Patel et al. [108] compared a number of fungal species when different types of lignocellulosic waste, i.e. wheat straw, rice straw, rice husk and bagasse were used for bioethanol production. *Aspergillus niger* and *Aspergillus awamori* led to the highest ethanol production form wheat and rice straw. On the other hand, the best results for rice husk and bagasse were achieved by *Aspergillus awamori* and *Pleurotus sajor-caju*, respectively.

Despite low energy consumption, modest environmental conditions and no chemical requirement, biological pretreatment still faces some drawbacks negatively affecting its widespread application as a commercial pretreatment method. These include long process time, large space requirement and the need for continuous monitoring of microorganism growth [109].

5. Combination of pretreatment methods

As mentioned earlier various pretreatment methods have some drawbacks limiting their applications. Combined pretreatment methods have been recently considered as a promising approach to overcome this challenge, by increasing efficiency of sugar production, decreasing formation of inhibitors and shortening process time. These would collectively result in higher bioethanol yield and more economical process.

5.1. Combination of alkaline and dilute acid pretreatments (ALK-DA)

As mentioned previously, acid pretreatment could delignify and increase the surface area of cellulose fiber. However, single-stage acid pretreatment with acetic acid for instance, requires much acid (50%, based on the initial quantity of the dry materials), so to overcome this challenge, a pre-pretreatment could be applied to partially remove lignin prior to this step. Alkali pretreatment has been known to be a suitable method for delignification and therefore, could be used in combination with acid pretreatment.

In a study, sugarcane bagasse was pretreated by alkali (NaOH)-peracetic acid (PAA) combined pretreatment under mild conditions. In short, 10% NaOH (based on the initial quantity of the dry materials) with 3:1 liquid-to-solid ratio at 90 °C for 1.5 h was

utilized in the first step. Then, 10% peracetic acid was employed at 75 °C for 2.5 h to delignify the regenerated biomass obtained in the first step. As a result, the yield of reducing sugars produced during the enzymatic hydrolysis (120 h, cellulase loading of 15 FPU/g solid) reached as high as 92.04%. Therefore, ALK-DA pretreatment (e.g. alkali-peracetic acid) in comparison with akali and acid pretreatment solely, could be performed under milder conditions while leading to more effective delignification and less carbohydrates degradation [48].

5.2. Combination of alkaline and IL pretreatments (ALK-IL)

Nguyen et al. [110] reported successful pretreatment of rice straw with the combination of ammonia and IL ([EMIM]Ac). This method is based on delignification effect of ammonia [109] and solubilization effect of ILs [13] in order to increase biodigestibility of lignocellulosic materials. The cellulose recovery and glucose conversion in this combined pretreatment has been found higher than those of the individual ammonia or ILs pretreatments. Moreover, higher pretreatment efficiency could be obtained by simplifying the sample communication, reducing the processing time for solubilization, using less enzyme amount for hydrolysis, and increasing the IL recycling rate for reuse.

5.3. Combination of dilute acid and steam explosion pretreatments (DA-SExp.)

The combination of dilute acid and steam explosion pretreatment (DA-SExp.) could lead to enhanced saccharification efficiency. Dilute-acid hydrolysis is the first step to maximize xylose conversion and steam explosion is the second step to break down the lignocellulosic structure. Recently, Chen et al. [30] investigated the application of DA-SExp. on rice straw with the first step (2% H2SO4) performed at 165 °C for 2 min followed by the second step performed at 180 °C for 20 min. Through the two-step pretreatment, a high xylose yield was obtained, and low levels of inhibitors were recorded. Based on their findings, a total of 75.9% xylan and 77.1% glucan were converted to xylose and glucose, respectively.

5.4. Combination of supercritical CO_2 and steam explosion pretreatments (SCCO$_2$-SExp.)

Comparison of supercritical CO_2 and combined supercritical CO_2 and steam explosion pretreatment (SCCO2-SExp.) of wheat straw was made by Alinia et al. [91]. The experimental results showed that the combined method conducted in two stages i.e. the steam-injection stage (200 °C for 15 min) and the supercritical CO_2 explosion (12 MPa, 190 °C for 60 min), was more efficient than the supercritical CO_2 (12 MPa, 190 °C and 30 min) alone. The reducing sugars obtained for the supercritical CO_2 and the SCCO$_2$-SExp. pre treatments were 149.1 and 234.6 g/kg wheat straw, respectively. Interestingly, wetting the feed prior to the supercritical CO_2, considerably increased this value to 208.4 g/kg wheat straw revealing the positive effect of wetting alone on the treatment procedure.

5.5. Combination of organosolv and biological pretreatments (bio-organosolv)

Bio-organosolv pretreatment was investigated by Monrroy et al. [111] to evaluate the synergic effect of the combination of the two methods on *P. radiata* wood chips. They used brown rot fungus *Gloephyllum trabeum* for 3 weeks on *P. radiata* followed by delignificatation of the biotreated material by organosolve ethanol:water mixture. The results obtained revealed that the same bioethanol yield was achieved for both experiments (i.e. bio-organosolv and organosolv alone); however, milder conditions (ethanol: water mixture (60/40 v/v), 185 °C, 18 min) were required when the chips had been initially biotreated in comparison to those of the organosolv pretreatment alone (ethanol: water mixture (60/40 v/v), 200 °C, 32 min). Simillar findings have been reported elsewhere when beech wood was subjected to biological pretreatment with white rot fungi i.e. *Ceriporiopsis subvermispora*, *Dichomitus squalens*, *Pleurotus ostreatus* (*P. ostreatus*), and *Coriolus versicolor* for 2–8 weeks fallowed by organosolv (ethanolysis) pretreatment [112]. The highest bioethanol yield was accomplished for *C. subvermispora*. Moreover, the combined pretreatment increased the pretreatment turnover up to 1.6 times and decreased the electricity requirement by 15% in comparison with the ethanolysis pretreatment alone.

These findings were also confirmed by those of Munoz et al. [113] where *P. radiata* and *Acacia dealbata* wood chips were biotreated with white rot fungi *C. subvermispora* and *Ganoderma australe* prior to organosolve pretreatment by ethanol–water mixture. The pretreatment conditions were 27 °C and 55% relative humidity for 30 days for the biological pretreatment and 60% ethanol solution at 200 °C and 1 h for the organosolve pretreatment. The experimental results showed an increased glucan production and reduced lignin content of theof wood chips treated by bio-organosolve in comparison with the sole organosolve pretreatment.

5.6. Combination of biological and dilute acid pretreatments (bio-DA)

Generally, acid pretreatment is a suitable method to dissolve hemicelluloses, while, biological pretreatment with fungas is known for disrupting the lignin–hemicellulose sheath, low energy requirements and mild environmental conditions [,]. Nevertheless, both pretreatment methods have some drawbacks such as being energy-intensive, environment-unfriendly and high pressure requirements for the acid pretreatment [114] and lack of economical feasibility and long process time for biological pretreatment. As a result, Fuying et al. [115] published a paper investigating the combination of biological and mild acid pretreatments (Bio-DA), in order to enhance enzymatic hydrolysis of water hyacinth. The combined pretreatment with Echinodontium taxodii and H2SO4 successfully led to enhanced enzymatic hedrolysis, elevated levels of reducing sugar and ethanol yield.

5.7. Combination of biological and steam explosion pretreatment (bio-SExp.)

Taniguchi et al. [116] used four white-rot fungi to pretreat rice straw and among the fungi used *P. ostreatus* was found to be most effective on degrading lignin; however, its impact on hemicellulose and cellulose degradation was low. Therefore, in order to decrease the considerable loss of holocellulose during the long pretreatment time (60 days) and to increase the efficiency of pretreatment, the combination with steam explosion (1.5 MPa for 1 min) was employed. Interestingly, the combination of the two methods reduced the pretreatment time significantly from 60 to 36 days while approximately the same net amount of glucose yield was achieved.

5.8. Microwave-assisted alkali pretreatment (MW-ALK)

As mentioned earlier, microwave irradiations could increase the chemical reaction rate through their synergic effect or in fact, leads to an explosion effect among the biomass particles. In a study, microwave-based heating was utilized instead of the conventional heating to pretreat switchgrass with an alkali. Under the

Table 2
Recombinant cell-wall-deconstructing enzymes in plants.

Plant	Objective	Transgenic enzyme	Subcellular storage compartment	References
Arabidopsis thaliana	Accumulation of endonuclease	*Acitothermus celluluolyticus* E1-CAT	Apoplast	[139]
	RBS promoter to enhancing endonuclease accumulation	*Thermotoga maritima* endoglucanase Cel5A	Chloroplast	[140]
	Xylan hydrolysis in biomass	*Dictyoglomus thermophilum* XynA and XynB	Apoplast	[141]
Tobacco	Xylan hydrolysis in biomass	*Clostridium thermocellum* XynZ	Cytosol, and ER	[142]
	Economic production of plant-derived enzyme	*C. thermocellum* *Cel*D and *Cel*O, *T. reesei* EgI, *Swo*I, *Axe*1, *Xyn*2 and B*gl*1, *Fusarium solani* *Pel*A, *Pel*B & *Pel*D	Chloroplast	[143]
	Mass production and autohydrolysis of endoglucanase	*T. maritima* endoglucanase, Cel5A, and CBM6-engineered Cel5A	Cytosol, apoplast, and chloroplast	[144]
	Endoglucanase expression	Thermostable E2, E3 and a E3-E2 fusion	Apoplast and cytosol	[145]
	Production of biomass hydrolyzing enzymes	*T. fusca* Thermostable cell wall-degrading enzymes	Chloroplast	[146]
	Enhancing hydrolysis of methylglucuronoxylans	*T. maritima* GH10 xylanase Xyl10B	Chloroplast	[147]
	Endoglucanase expression	*A. cellulolyticus* E1-CAT	Chloroplast	[148]
	Endoglucanase expression	*T. fusca* Cel6A gene encoding an endoglucanase	Chloroplast	[149]
	Production of multifunctional lignocellulosic hydrolases			[150]
	Enhancing cellulase activity	*A. celluluolyticus* E1 and E1CAT	Apoplast	[151]
	Enhancing cellulase activity	*A. cellulolyticus* Cel5A	Targeted to the cell wall	[152]
	Enhancing cellulase activity	*A. celluluolyticus* E1	ER	[153]
	Hemicellulose hydrolysis	*C. thermocellum* XynZ	Apoplast	[154]
	Enhancing cellulase activity	*A. celluluolyticus* E1	Chloroplast	[153,155]
	Enhancing cellulase activity	Maize β-glucosidase	Chloroplast	[156]
	Enhancing cellulase activity	*A. celluluolyticus* E1 CAT	Chloroplast	[151]
	Enhancing cellulase activity	*T. fusca* E2 and E3	Cytosol	[157]
	Enhancing cellulase activity	*T. reesei* CBH1	Cytosol	[158]
	Enhancing cellulase activity	Human β-glucosidase	Cytosol	[159]
	Hemicellulose hydrolysis	*C. thermocellum* XynA CAT	Cytosol	[160]
	Enhancing cellulase activity	*A. cellulolyticus* E1	Cytosol	[88]
Potato	Enhancing cellulase activity	*A. celluluolyticus* E1	Apoplast, chloroplast, vacuole	[161]
	Enhasing xylan hydrolysis	*Streptomyces olivaceoviridis* XynB	Apoplast	[162]
	Enhancing cellulase activity	*T. fusca* E2	Cytosol	[163]
	Enhancing cellulase activity	*T. fusca* E3	Cytosol	[157]
	Hemicellulose hydrolysis	*S. olivaceoviridis* XynB	Cytosol	[164]
Alfalfa	Enhancing cellulase activity	*T. fusca* E2 and E3	Cytosol	[157]
Rice	Rice biomass hydrolysis	*A. celluluolyticus* E1-CAT	Apoplast	[165]
	Hemicellulose hydrolysis	*C. thermocellum* XynA CAT	Cytosol	[160]
Barley	Hemicellulose hydrolysis	Rumen *Neocallimastix patriciarum* XynA	Cytosol	[166]
Sugar cane	Sugar cane biomass hydrolysis	CBH I, CBH II, EG	Tissue specific expression	[167]
Maize	Hydrolysis of maize biomass	*A. cellulolyticus* E1	ER and mitochondria	[168]
	Corn stover biomass hydrolysis	XynA, XynB, *Nasutitermes Takasagoensis* EGA, *A. cellulolyticus* EGB, *Aspergillus niger* AccA, AccB, AccA/AccB	Apoplast	[169]
	Hydrolysis of maize biomass	*A. celluluolyticus* E1CAT	Apoplast	[170]
	Hydrolysis of maize biomass	*A. cellulolyticus* E1, *T. reesei* CBH I, exo-cellulase and bovine rumen *Butyrivibrio fibrisolvens* cellobiase	ER, apoplast, vacuole	[171]
	Enhancing cellulase activity	*A. cellulolyticus* Cel5A	Targeted to the cell wall	[152]
	Hemicellulose hydrolysis	Xylanase	Chloroplasts	[172]
	Hydrolysis of maize biomass	*A. celluluolyticus* E1-CAT	Apoplast	[173]
Sengon	Hydrolysis xyloglucan bounds	Poplar cellulose	Apoplast	[174]

AccA, AccB, AccA/B: feruloyl esterase A, B, A/B, CAT: catalytic domain, CBH1, CBHII: celluobiohydrolase, EGA and EGB: endo-β-1,4-glucanase, cel: endocellulase, E1, E2 and E3: endoglucanases (endocellulases), ER: endoplasmic reticulum, *Pel*A, *Pel*B and *Pel*D: pectate lyase, XynA, XynB and XynZ: xylanases (hemicellulases).

optimum temperature of 190 °C and 50 g/L solid content for 30 min treatment time, the sugar yield recorded was 58.7 g/100 g biomass [40]. In another study, Shengdong et al. [117] evaluated the effect of MW-ALK pretreatment (700 W) on wheat straw, and the results were compared with those of the conventional alkali pretreatment (1% NaOH). The cellulose content of 79.6 vs. 73.5%, lignin content of 5.7 vs. 7.2%, hemicellulose content of 7.8 vs. 11.2% and reaction time of 25 vs. 60 min were recorded after the MW-ALK and alkali pretreatments, respectively. In conclusion, the MW-ALK pretreatment resulted in more lignin and hemicelluloses removal from wheat straw and shorter pretreatment time. Moreover, the hydrolysis rate of the combined method was higher than that of the conventional alkali pretreatment. Similar positive findings were previously reported by Shengdong et al. [118] where they applied the same combined method on a different feedstock i.e. rice straw.

5.9. *Combination of dilute acid and microwave pretreatments (DA-MW)*

In a study, the impact of dilute sulfuric acid pretreatment on sugarcane bagasse combined with microwave heating was evaluated [119]. In that study, 10 g of bagasse sample (on a dry basis) was soaked in 200 ml acid sulfuric with concentration of 1.56 wt %,

blended in a reactor and subjected to heating by microwave at three different reaction temperatures of 130, 160, 190 °C and two heating times of 5 and 10 min. At 190 °C, highest biomass fragmentation and swelling as well as complete hemicellulose degradation were achieved. However, increasing time had no significant effect on the contents of the constituents.

5.10. Combination of IL and ultrasonic pretreatment (IL-UL)

The application of ultrasound as a pretreatment method instead of the conventional heating pretreatment could lead to the enhancement of the saccharification ratio. In a study, Ninomiya et al. [120] evaluated the synergic effect of the combination of ultrasonic pretreatment and different ILs i. e. [BMIM]Cl, [AMIM]Cl, [EMIM]Cl, [EMIM]DEP, and [EMIM]Ac. In their report, the application of conventional heating at 110 °C for 120 min for kenaf powders pretreated in firsr four ILs mentioned above resulted in the cellulose saccharification ratio of about 20%. This was significantly less than the ratio of 60–95% obtained when the conventional heating was replaced by the ultrasonic pretreatment in the same ILs at 25 °C for 120 min. Surprisingly, the cellulose saccharification ratio of kenaf powder in [EMIM]Ac was as high as 86% after only 15 min of the ultrasonic pretreatment at 25 °C, compared to only 47% in the case of thermal pretreatment in the same IL.

6. Future prospective of pretreatment; genetic manipulation of energy crops

It has been estimated that about 18–20% of the total projected cost for biological production of lignocellulosic ethanol can be attributed to pretreatment, more than for any other single steps [121,122]. Genetic and metabolic engineering could also play a crucial role in facilitating pretreatment and hydrolysis processes and consequently in economical production of ethanol from lignocellulosic wastes. Currently, different omicses tools (functional genomics, metagenomics, transcriptomics, proteomics and metabolomics), high throughput sequencing and genetic engineering strategies are used to enhance pretreatment and hydrolysis of lignocelulosic biomasses. In this section a summary of different omices and genetic engineering (upstream) approaches used to enhance economic plant biomass hydrolysis is reported.

Recent advances in understanding the biochemical machinery used by different plants in cell wall structure and their biochemical characteristics, such as cellulose, hemicelluloses and lignin offers new avenues for the development of new biological-based processes for biomass conversion to bioethanol at industrial scale. Whole genome sequences of different plants, such as maize, rice, sugar cane and barley has accelerated this process [123,124]. As discussed earlier, cellulose-crystallinity and lignin cross linking are known as major barriers critically making biomass pretreatment and enzyme digestion expensive [124]. Therefore, altering plant cell wall structure toward appropriate traits for bioethanol production, such as high cellulose content, low lignin content and recalcitrance as well as high hydrolase activity, is a key step for improving biomass quality. It is important to note that because of the diversity in plant cell wall structure and the complexity of its function, the genetic modification of plant cell walls could unexpectedly lead to alteration of plant cell growth and development [125]. Jensen et al., [126], studied the genetic variation in degradability of some wheat varieties straw and the improvement potentials through plant breeding. They showed that degradability of cereal straw was not correlated with grain yield, and therefore straw degradability may be improved through breeding without any serious negative effects on grain yield. Also, Xie and Peng [124] reported increasing biomass degradability of rice through mutagenesis.

Previously, mutation breeding and marker assisted selection methodologies have been widely used in plant improvement programs for crop yield, plant biotic and abiotic resistance, as well as quality. Recently, the genetic engineering of the selected energy crops and their cellulosic biomass has been taken in account for further increasing biomass yield and bioethanol production at large scale. Brereton et al. [127] studied the identified quantitative trait loci (QTL) associated with enzymatic saccharification yield in willow varieties. They found significant natural variation in glucose yields from willow stem biomass, and four enzyme-derived glucose QTL were mapped onto chromosomes V, X, XI, and XVI, indicating that enzymatic saccharification yields are under significant genetic influence.

These strategies rely on genetic modification of plant cell walls by specifically altering wall polymer inter-linking and cellulose crystallinity, reducing lignin and phenolic acid ester levels, increasing specific hemicelluloses contents, and adding foreign cellulase enzymes and/or other wall proteins. To achieve the above goals, selection of appropriate genes is an initial and crucial step, and the related genetic manipulation approach should then be considered [128]. Fortunately, genetic engineering of most food crop and woody plant species, such as rice, corn, wheat, barley, sorghum, poplar, willow, switch grass has been well established, using either *Agrobacterium tumefaciens* or gene-gun-mediated gene transfer [129]. The most important plant genetic engineering strategies to enhance economic pretreatment and hydrolysis of lignocellulosic biomass are presented below.

6.1. Increasing cellulose composition

One of the most important strategies to reduce the pretreatment cost is by increasing cellulose content of plant biomass. So far, more than 1000 genes have been found to be related to plant cell wall biosynthesis, degradation and regulations and the search for finding new candidate genes involved in cell wall and cellulose biosynthesis is on-going [125]. Two major gene superfamilies *ces*A and *csl* involved in cellulose biosynthesis have been identified in rice, maize and other crops, which could be considered for biomass enhancement [124]. In addition to these two genes, some other genes, such as *Korrigan*, *Cobra* and *Kobito*, involved in cell wall synthesis could also be used for energy crop modification [130]. Importantly, as major transcription factors are identified for regulating secondary cell wall synthesis in *Arabidopsis*, it is possible to directly improve quantity and quality of biomass by altering the expression time and level of these genes in energy crops. Coleman et al. [131] showed that overexpression of the gene *SuSy* in poplar led to cellulose content increase by 2–6% without any negative consequences on plant growth habits. It has been suggested that the overexpression of this gene in other plants may also significantly enhance the cellulose content of the plant biomass [124].

6.2. Reduction of plant cell wall recalcitrance and cellulose crystallinity

One of the challenges faced during the pretreatment of lignocellulosic biomasses is plant cell wall recalcitrance due to the extreme complexity of the cell-wall matrix, which causes difficulty in biomass degradation. The plant cell wall recalcitrance is a direct function of cellulose crystallinity leading to plant resistance to biotic and abiotic stresses. Plant cell wall recalcitrance also depends on the types of hemicelluloses and the ratio of lignin monomers [14]. There are two major hemicelluloses in grasses: β-1,3-β-1,4-glucan and β-1,4-linked xylose backbone with single arabinose and glucuronic acid side chains [132]. To overcome this barrier for economic bioethanol production, it is feasible to genetically introduce some special microorganism-derived

cellulose binding proteins and enzymes into cell walls. Fry et al. [133] managed to reduce recalcitrance by increasing the ratio of β-1,3-β-1,4-glucan backbone by over-expressing *cslF* and *cslH* genes that have been characterized to catalyze β-1,3-β-1,4-glucan biosynthesis. In addition to this, it was also found possible to down-regulate three glycosyltransferase (TaGT) proteins participating in the β-1,4-linked xylose polymer synthesis, and as result to reduce the level of plant biomass recalcitrance [134].

On the other hand, reduced cellulose crystallinity could be achieved by the expression of cellobiose dehydrogenase in feedstock crops. Previously, it has been reported that cellobiose dehydrogenase in a crude mixture of cellulases could increase solubility of crystalline cellulose [135].

This may be because of preventing the re-condensation of glycosidic bonds of cellulose chains that have been nicked by endocellulases and consequently changing the structure of cellulose, hemicellulose and lignin by creating hydroxyl radicals. In addition, a number of studies showed that transferring α-glucosidase and β-glucosidase into plants increased the cellulose solubility and facilitated the conversion of plant tissues into fermentable sugars [136,137].

6.3. Production of hydrolases in plants

During the bioconversion process of lignocellulosic biomass to bioethanol, the hydrolysis of cell wall lignocellulose is synergistically catalyzed by cellulases, including endoglucanases, exoglucanases and β-glucosidases [7,55,110]. At present, these plant cell-wall hydrolyzing enzymes are expensively produced in microbial bioreactors for commercial use. On the other hand, plants are already used industrially for the production of enzymes and other proteins, carbohydrates, lipids, industrial polymers and pharmaceuticals. Moreover, expertise is available for plant genetic transformation, farming of transgenic crops as well as harvesting, transporting and processing the plant matter. Cell-wall hydrolyzing enzymes can potentially be produced in all feedstock crops that are to be used for cellulosic ethanol production.

The plant-based production of these enzymes has a crucial advantage i.e. growing transgenic plants in the field requires a much lower energy input than that of the microbial production of these enzymes. Since many of the cell-wall hydrolyzing enzymes identified so far are of bacterial origin, codon alteration of the coding region is usually necessary to ensure efficient expression in plants; this is a straightforward procedure that is widely used for the heterologous expression of microbial proteins in eukaryotes. Another potential concern is misfolding of the enzymes in their new environment [138]. Over the past years, there have been several attempts to express microbial cellulase genes in plants, and to determine their hydrolysis activity in the transgenic plants, such as tobacco, maize, potato, sugar cane, barley, arabidopsis, sengon, rice and alfalfa (Table 2)[88,139–174]. Interestingly, no visible side-effect on plant growth and biomass yield was observed [124]. Gadab et al. [175] expressed the catalytic domain of the thermostable 1,4-b-endoglucanase (E1) of *A. cellulolyticus* in corn and proved that this crop could be used as a bioreactor for cellulose-degrading enzymes.

When expressed in plants, accumulation of the cell-wall hydrolyzing enzymes in subcellular compartments is preferred over their accumulation in cytosol. When targeted for accumulation in subcellular compartments, including the apoplast, vacuole, endoplasmic reticulum, Golgi apparatus, and microbodies such as liposomes and peroxisomes, these enzymes are more likely to display correct folding and activity, glycosylation, reduced degradation and increased stability, as compared to production and accumulation in the cytosol [138,176]. The subcellular targeting of heterologously-expressed hydrolyzing enzymes is important for several reasons; first such targeting keeps the foreign enzymes away from cytoplasmic metabolic activities, avoiding potential damage. Secondly, it also allows higher levels of enzyme accumulation and can increase enzyme stability through reduced exposure to proteases. Finally, targeting can also enable better folding of proteins in subcellular compartments where there are molecular chaperones, and keeps the cell-wall hydrolyzing enzymes away from host cell walls.

Several microbial hydrolyzing enzymes have already been produced in plants through subcellular targeting. However, most previous research has been performed on tobacco and alfalfa, which are not biofuel crops. Recently, a great deal of research endeavors on biofuel crops, such as maize, potato, barley, rice and sugar cane has been started. As indicated above, the cytosol might not be an ideal location for the accumulation of heterologous molecules because of potential interference with metabolic activities. Therefore, the apoplast has been selected in many cases, assuming that this compartment is the most spacious and thus, capable of accumulating large quantities of heterologous proteins [139,172]

In addition to corn, the E1 enzyme originally obtained from *Acidothermus cellulolyticus* has been produced in rice at almost 5% plant total soluble protein (TSP), but these levels need to be increased to about 10% TSP for complete hydrolysis without the need for addition of microbially produced endoglucanase. It has been shown that expressing just the catalytic domain of these enzymes results in a higher level of expression [168]. Another method of increasing the level of enzyme production is to genetically engineer the chloroplast genome instead of the nuclear genome. Because the chloroplast genome of most flowering plants is maternally inherited, chloroplast transgenesis also provides the benefit of transgene containment, which is important for crops with out-crossing wild relatives. Genetic transformation of chloroplast genomes is now possible in most dicotyledonous crops, including poplar [88,165,173]. In arabidopsis, when a heterologous fungal xylanase was targeted to either the chloroplast, the peroxisome or both of these compartments, the dual compartment targeted xylanase accumulated 160% of that targeted to the chloroplast alone and 240% of that targeted to the peroxisome alone [168,172].

6.4. Lignin modification

Three precursors, including paracoumaryl, coniferyl and sinapyl alcohols are involved in lignin biosynthesis. The structure of lignins is composed of guaiacyl (35%–49%), syringyl (40%–61%) and hydroxycinnamates (4%–15%) units. The ferulic acid and coumaric acid are also present in plant cell walls [177,124]. The most important factors in the level of lignocellulose biodegradation are the rate of lignins and phenolic acids esters, and the ratio of coniferyl lignin to syringyl lignin. The rate of esterified phenolic acids, including the ferulic and p-coumaric acids, are also key factors which limit biodegradation of nonlignified cell walls in grasses [178,179].

To decrease the lignin level in the lignocellulosic biomass, at first it is necessary to select and characterize the major genes involved in lignin biosynthesis pathways. If a gene is a housekeeper, it should be partially silenced by RNA interference (RNAi) technology rather than totally knocked out by antisense [124]. Several lignin metabolism key enzymes, such as cinnamate 4-hydroxylase (C4H); hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT); coumaroyl shikimate 3-hydroxylase (C3H); caffeoyl CoA 3-O-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H); or caffeic acid 3-O-methyltransferase (COMT), have been characterized in dicot plants, so down regulation of these key enzymes in order to modify the chemical structures of

Table 3
Plant genetic engineering to change lignin features.

Plant	Aim	Gene	Reference
Wheat	Lignin reduction	Down regulating cinnamoyl-CoA reductase and low phytic acid	[181]
Barley	Lignin reduction	Down regulating cinnamoyl-CoA reductase and low phytic acid	[181]
Alfalfa	Lignin reduction and enhance hydrolysis	Down regulation of the caffeic acid O-methyltransferase	[182]
	Lignin reduction	Cytochrome P450 enzyme	[183]
	Lignin reduction	Cinnamyl alcohol dehydrogenase (CAD)	[184]
	Lignin reduction and increase fermentable sugars	Cinnamate 4-hydroxylase (C4H); hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (HCT); coumaroyl shikimate 3-hydroxylase (C3H); caffeoyl CoA 3-O-methyltransferase (CCoAOMT); ferulate 5-hydroxylase (F5H); or caffeic acid 3-O-methyltransferase (COMT)	[185] [179]
	Lignin reduction	Down-regulation of coumarate 3-hydroxylase (C3H)	[155]
	Lignin reduction	Cinnamoyl CoA reductase (CCR) or cinnamyl alcohol dehydrogenase (CAD)	[186]
Tobacco	Lignin reduction	Down-regulation of *Leucaena leucocephala* cinnamoyl CoA reductase (LlCCR) gene	[187]
	Biomass increase	O-methyl transferase (OMT)	[188]
	Lignin reduction and xylose and glucose increase	Cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD)	[189]
Pinus radiata	Lignin reduction	Caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT)	[190]
Sweet sorghum	Lignin reduction	Down-regulation caffeoyl CoA-O-methyltransferae (CCoAOMT)	[191]
Poplar	Change the structure of lignin	Overexpression of the ferulate 5-hydroxylase (F5H) gene	[192]
Arabidobsis	Change the structure of lignin	Mutation in the gene encoding caffeic acid *O*-methyltransferase (*comt*) with over-expression of ferulate 5-hydroxylase (*F5H1*)	[193]
	Change the structure	Catabolic enzyme LigD	[194]
	Increase 5-hydroxy-guaiacyl units	Over-expressing ferulate 5-hydroxylase and downregukating lacking caffeic acid *O*-methyltransferase.	[195]
Switchgrass	Lignin reduction	Silencing (RNAi) of 4-coumarate:coenzyme A ligase (4CL)	[196]
Populus sp.	Lignin reduction	Cinnamyl alcohol dehydrogenase (CAD)	[197]
Maize	Lignin reduction	O-methyl transferase (OMT)	[198]
	Lignin reduction	O-methyl transferase (OMT)	[199]
Aspen	Lignin reduction	4-coumarate CoA ligase (4CL)	[200]
	Lignin reduction	CAld5H and 4CL	[201]
	Change the structure of lignin	Catabolic enzyme LigD	[194]

Table 4
Effect of different pretreatment methods on the chemical composition and structure of lignocellulosic biomass and their limitation.

Pretreatment method	Increase specific surface	Cellulose de-crystallization	Hemicellulose removal and solubilization	Lignin removal	Inhibitor compounds formation	Drawback and disadvantages
Physical	++[a]	++	–	–	–	High energy consumption
Acid	++	–	++	+	++	Equipment corrosion, degrading produce sugar, neutralization of pretreated slurry
Alkaline	++	–	+	++	+/–	Long pretreatment resident time, neutralization of pretreated slurry
Ionic liquid	++	++	+	+	–	High cost of ionic liquid
Organosolv	++	ND	++	++	–	Recovery and recycle of solvent by evaporation, high cost
Ozonolysis	++	ND	–	++	–	Large amount of ozone requirement, expensive process
Steam explosion	++	–	++	+/–	++	Incomplete disruption of lignin–carbohydrate matrix, formation of toxic component
AFEX	++	++	+	++	+/–	High pressure requirement, low efficiency for high lignin content biomass, high cost of ammonia
Co_2 explosion	++	–	++	–	–	High pressure requirement, does not affect on lignin and hemicelloluse
Wet oxidation	++	+	++	++	+/–	High cost of oxygen and catalyst
LHW	++	ND	++	+/–	+	High temperature, need to add alkaline to control PH
Biological	++	+	+/–	++	–	Low hydrolysis rate, large space requirement, watchful control condition of microorganism growth

[a] ++: high effect; +: moderate effect; +/–: low effect; N.D: not determined.

lignin components and/or reduce plant lignin content is a potentially promising way to reduce pretreatment costs in bioethanol production [179,180].

The research works dedicated to change lignin features in plants are summarized in Table 3 [155,179,181–201]. Down-regulation of lignin biosynthesis enzymes was initially performed using antisense oligonucleotides; however, RNAi technology has been also used for this purpose [155]. Down-regulation of some key enzymes, such as C3H, cytochrome P450 enzyme, cinnamyl alcohol dehydrogenase (CAD), O-methyl transferase (OMT) in different plants such as alfalfa, wheat, barley, corn, populous sp., *Pinus radiata* and tobacco resulted in modification of lignin residue composition and increased in situ digestibility (Table 4).

Shifting energy from lignin biosynthesis to polysaccharide synthesis is another strategy to reduce the lignin content of biomass. For example, down-regulation of 4-coumarate CoA ligase (4CL), coniferaldehyde 5-hydroxylase (CAld5H) and cinnamoyl CoA reductase (CCR) in transgenic plants (corn, aspen, switch

grass, arabidopsis) resulted in a decrease in lignin content and a concomitant increase in xylose and glucose associated with the cell wall [179,196]. For efficient and sustainable production of kraft pulp and the other biomass-derived products such as bioethanol, Ishikawa et al. [194] successfully transfered the *lig*D gene into arabidopsis and hybrid aspen and managed to generate transgenic plants whose lignin could be easily removed from hollocellulose fraction under alkaline conditions.

6.5. Protein modules disrupting plant cell wall substrates

Some protein modules, such as expancins and swollenin, have been recognized to disrupt plant cell-wall substrates, potentially by increasing the accessibility and efficiency of hydrolases. Previously, the important role of expansins in loosening the cell wall to allow expansion and growth, and subsequently increase in cellulose deconstruction efficiency has been documented [202,203]. In addition, it was shown that 'swollenin' has a cellulose-binding domain and an expansin-like domain, which together have a disrupting effect on crystalline carbohydrates [204]. Transferring or over-expression of these genes in the cellulosic biomass crops might provide another route towards modifying cell walls and decreasing the need for expensive pretreatment processes.

7. Conclusion

Lignocellulosic biomass as an available and cheap source is gaining popularity as a source of fermentable sugars for liquid fuel production. One of the most expensive steps of bioethanol production from such biomass is pretreatment followed by enzymatic treatment. Extensive research has been carried out in order to increase fermentable carbohydrate recovery, decrease inhibitors produced from sugar degradation during pretreatment process, diminish utilization of chemical materials and energy input, produce valuable by-products and decrease cost of bioethanol process. In general, pretreatment technologies are divided into four major groups i.e. physical, chemical, physic-chemical and biological. Although each method has some advantages, one method could not be the choice for all types of biomass. Effects of different pretreatment methods on the chemical composition and structure of lignocellulosic biomass and their limitation are presented in Table 4. Fundamental understanding of various pretreatment technologies, different composition of biomass feedstock and the relationship between composition of biomass feedstock and pretreatment methods would significantly help in matching the best pretreatment method/combinations for a specific biomass feedstock. On the other hand, recent advances in functional genomics, metagenomics, genetic and metabolic engineering imply that the future of economic bioethanol production form biomass will strongly depend on achievements in artificially-designed plants, containing high levels of cellulose while capable of producing hydrolases.

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